Pharmacological Preconditioning of Mesenchymal Stem Cells with Trimetazidine (1-[2,3,4-Trimethoxybenzyl]piperazine) Protects Hypoxic Cells against Oxidative Stress and Enhances Recovery of Myocardial Function in Infarcted Heart through Bcl-2 Expression

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ABSTRACT

Stem cell transplantation is a possible therapeutic option to repair ischemic damage to the heart. However, it is faced with a number of challenges including the survival of the transplanted cells in the ischemic region. The present study was designed to use stem cells preconditioned with trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine; TMZ), a widely used anti-ischemic drug for treating angina in cardiac patients, to increase the rate of their survival after transplantation. Bone marrow-derived rat mesenchymal stem cells (MSCs) were subjected to a simulated host tissue environment by culturing them under hypoxia (2% O_2) and using hydrogen peroxide (H_2O_2) to induce oxidative stress. MSCs were preconditioned with 10 μM TMZ for 6 h followed by treatment with 100 μM H_2O_2 for 1 h and characterized for their cellular viability and metabolic ac-tivity. The preconditioned cells showed a significant protection against H_2O_2-induced loss of cellular viability, membrane dam-age, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, phosphorylated Akt (pAkt), and Bcl-2 protein levels and Bcl-2 gene expression. The therapeutic efficacy of the TMZ-preconditioned MSCs was evaluated in an in vivo rat model of myocardial infarction induced by permanent ligation of left anterior descending coronary artery. A significant increase in the recovery of myocardial function and up-regula-tion of pAkt and Bcl-2 levels were observed in hearts trans-planted with TMZ-preconditioned cells. This study clearly demonstrated the potential benefits of pharmacological pre-conditioning of MSCs with TMZ for stem cell therapy for repair-ing myocardial ischemic damage.

Myocardial infarction (MI) is a major contributor to chronic heart disease leading to mortality in humans. Transplanta-tion of stem cells (cellular cardiomyoplasty or cell therapy) in the infarcted myocardium has been considered a possible therapeutic option to repair the infarcted myocardium and restore the function of the damaged heart (Dimmeler et al., 2008). A variety of cells including embryonic stem cells, fetal cardiomyocytes, cardiac stem cells, skeletal myoblasts, smooth muscle cells, hematopoietic stem cells, or mesenchy-mal stem cells are being explored as potential choices for myocardial cell therapy (Dimmeler et al., 2008). Mesenchy-mal stem cells (MSCs), which are self-renewing precursor cells of nonhematopoietic stromal tissues, are currently un-der intense investigation for cardiac repair (Nesselmann et al., 2008). MSCs are adult pluripotent cells, which can be isolated from bone marrow and other adult tissues and easily propagated in vitro (Pittenger et al., 1999). These cells can be directed to differentiate into osteoblasts (Heino and Hen-tunen, 2008), chondrocytes (Pereira et al., 1995), vascular endothelial cells (Yue et al., 2008), or cardiomyocytes (Toma

ABBREVIATIONS: MI, myocardial infarction; MSC, mesenchymal stem cell; TMZ, trimetazidine, 1-[2,3,4-trimethoxybenzyl]piperazine; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; LDH, lactate dehydrogenase; H_2O_2, hydrogen peroxide; PI, propidium iodide; OCR, oxygen consumption rate; EPR, electron paramagnetic resonance; pAkt, phosphorylated Akt; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAD, left anterior descending (coronary artery); LV, left ventricle; EF, ejection fraction; FS, fractional shortening.

et al., 2002) using specific growth factors and conditions. Furthermore, MSCs can suppress local inflammation (Djouad et al., 2003) and trigger local production of growth factors and cytokines favoring endogenous cardiac repair. Thus, MSCs appear to be an ideal cell choice for myocardial tissue repair.

Stem cell transplantation to the infarcted myocardium is faced with additional challenges beyond finding the ideal cell type for use. The infarct region is usually ischemic, with the development of a scar tissue that may not facilitate the transport of essential nutrients and oxygen to support the engraftment and survival of the transplanted stem cells. Most of the cells die within hours of transplantation in the infarcted heart because of interplay of ischemia, inflammation, and apoptosis (Menasché, 2008). Several strategies have been proposed to improve revascularization of the ischemic tissue or to enhance the longevity of the transplanted cells in the hostile ischemic environment. For example, preconditioning the stem cells using chemokines, growth factors, or pharmacological agents has been shown to improve their survival at the site of transplantation (Shmelkov et al., 2005; Niagara et al., 2007; Pasha et al., 2008).

Oxygen is an essential metabolic substrate required for the production of energy to support the survival, proliferation, and differentiation of the transplanted cells in the infarct myocardium. Under aerobic conditions, cellular energy production (ATP) involves predominantly fatty acid oxidation pathway, which is oxygen intensive. However, under hypoxic conditions, which occur in the infarct myocardium, it would be advantageous for cells to switch to alternate pathways, such as anaerobic glycolysis, for energy production, thereby reducing dependence on tissue oxygenation. In the clinical setting, this is usually achieved through the application of anti-ischemic drugs, such as trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine; TMZ; also known as Vastarel in the United States), which is used to reduce ischemia-induced metabolic damage by lowering the tissue demand for oxygen (Lopaschuk et al., 2003). TMZ reduces the rate of free fatty acid oxidation, with a concomitant increase in anaerobic glucose oxidation rates during low-flow ischemia (Kantor et al., 2000). The likely mechanism of TMZ action is through the inhibition of 3-ketoacyl CoA thiolase enzyme, which is crucial to the β-oxidation of fatty acids. Thus, inhibition of the fatty acid oxidation pathway by TMZ appears to be a clinically relevant solution to compromise the reduced supply of oxygen to the ischemic heart tissue. However, the pharmacological efficacy of anti-ischemic agents, such as TMZ, in augmenting myocardial stem cell therapy has not yet been reported.

Therefore, the goal of the present study was to investigate whether pharmacological preconditioning of MSCs with TMZ could enable them to survive in the hypoxic environment in the infarct tissue upon transplantation. Bone marrow-derived rat MSCs were subjected to a simulated host tissue environment by culturing them under hypoxic conditions and inducing oxidative stress using hydrogen peroxide. These cells were further treated with TMZ and characterized for their cellular viability and metabolic activity. The therapeutic efficacy of the TMZ-preconditioned cells was studied in an in vivo rat model of myocardial infarction. The results clearly demonstrated the potential benefits of TMZ in preconditioning MSCs before implantation to offer a significant enhance-

ment in the functional recovery of infarcted myocardium with an insight into the mechanism of action.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycin, trypsin, sodium pyruvate, and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) colorimetric assay kit, lactate dehydrogenase (LDH) assay kit, antibodies against β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies specific to HIF-1α, survivin, Bcl-2, Akt, and phospho-Akt were obtained from Cell Signaling Technology Inc. (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence kit was obtained from GE Healthcare (Chalfont St. Giles, UK). The bicinchoninic acid protein assay kit was obtained from Pierce Chemicals (Rockford, IL). Polyvinylidene fluoride membrane and molecular weight markers were obtained from Bio-Rad (Hercules, CA). TMZ was synthesized as reported (Kálai et al., 2006).

In Vitro Experiments. Rat MSCs were purchased from Millipore Biosciences Research Reagents (Temecula, MA). The cells were cultured using Dulbecco's modified Eagle's medium with GlutaMax 1 (4500 mg/l glucose) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Aerobic cultures of cells were maintained using 5% CO_2 in air (20% O_2) at 37°C in a humidified incubator. Hypoxic cultures were performed using cells of third or fourth passages at ~80% confluence. The cells were incubated in serum-free media under hypoxic conditions (2% O_2, 5% CO_2, balance N_2) using a BioSpherix (Redfield, NY) growth chamber for 12 h before treatments. The 2% O_2 was chosen to provide a sublethal dose of hypoxia corresponding to the levels in the ischemic heart, which ranges from 0.2 to 2.6% O_2 (Khan et al., 2007). The cells, under hypoxic conditions, were treated with TMZ (10 μM) for 6 h followed by H_2O_2 (100 μM) for 1 h to induce oxidative stress. The optimal dose of TMZ and H_2O_2 and treatment periods were determined from a preliminary dose/time study.

Cell Viability Using Propidium Iodide Binding Assay. The nuclear viability of the cells was measured using an automated cell counter (NucleoCounter; New Brunswick, Edison, NJ). This technique uses propidium iodide (PI), which binds to cellular nuclei. Depending upon sample preparation, the counts provide the total number of cells and number of nonviable cells, from which the number of viable cells is calculated.

Cell Viability (Mitochondrial Activity) by MTT Assay. The effect of TMZ on the mitochondrial activity of MSCs was determined by MTT assay using MTT colorimetric assay kit. Cells were seeded in 96-well plates at a concentration of 5×10^5 cells/well in a 200-μl volume of growth medium. After treatment period, the supernatant was removed, washed three times with 1× PBS, and 200 μl of MTT reagent in plain RPMI-1640 medium was added. The plates were incubated for 4 h at 37°C in a humidified incubator. The MTT reagent solution was removed from each well, and acidified-methanol was added to dissolve the formazan salt. The plates were then loaded into an automated plate reader (Beckman Coulter, Fullerton, CA; AD340) and analyzed at λ = 490 nm to determine the quantity of formazan product present in each well. All assays were run in at least three parallels and repeated three times.

Cytotoxicity Using LDH Assay. After treatment, MSCs were washed, trypsinized, centrifuged, and resuspended in 15 ml of growth medium without serum. Cytotoxicity was determined by measuring the quantity of LDH found in the culture medium using a standardized LDH assay. The culture supernatants from each experiment were collected and stored at −80°C until the assays were performed. Samples were thawed, and LDH assays were performed at 25°C. Readings were taken at 340 nm using a Varian (model Cary

50; Palo Alto, CA) spectrophotometer. All assays were run in at least three parallels and repeated three times.

Oxygen Consumption Rate Measurement. The effect of TMZ on cellular mitochondrial activity was assessed by measuring the oxygen consumption rate (OCR) using electron paramagnetic resonance (EPR) oximetry as reported previously (Pandian et al., 2003; Wisel et al., 2007). At the end of each treatment period, the cells were washed, trypsinized, and resuspended to a final concentration of 1×10^6 cells/ml in PBS. A 30-μl cell suspension was loaded into a microcapillary tube, and both ends of the tube were sealed. EPR measurements were performed using a Bruker X-band (9.8 GHz) spectrometer (Bruker BioSpin; Newark, DE). OCRs were determined from a series of EPR spectra measured for 30 min (Pandian et al., 2003; Wisel et al., 2007).

Western Blot Analysis. MSCs were treated with radioimmuno-precipitation assay buffer to lyse the cells. The total protein concentration within the samples was determined using a bicinchoninic acid protein assay kit. Twenty micrograms of protein was resolved by electrophoresis in a 10% SDS gel for 3 h and transferred to a polyvinylidene difluoride membrane overnight at 4°C. The membranes were blocked in PBS buffer containing 0.2% Tween 20 and 5% nonfat milk for 2 h at room temperature. The blots were then incubated overnight at 4°C with antibodies specific to HIF-1α, survivin, Bcl-2, Akt, or phosphorylated Akt (pAkt). β-Actin was used as a loading control. Primary antibody binding was detected with horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence kit. The intensity of the bands from each protein under investigation was quantified using ImageQuant software.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was isolated from MSCs using the RNeasy kit from QIAGEN (Valencia, CA). RNA quantification was done using spectrophotometry. Reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis for the mRNA expressions in Bcl-2 and the internal control GAPDH was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA), under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s), and extension at 72°C for 5 min. The sequences (5′-3′) for the primer pairs of Bcl-2 and GAPDH were as follows: Bcl-2, AGGATTGTGGCCTTCTTTGAG (forward) and GAGACAGCCAGGAGAAATCAAA (reverse); and GAPDH, GTCAACGGATTTGGTCGTATT (forward) and AGTCTTCTGGGTGGCAGTGAT (reverse). The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide.

In Vivo Experiments. A rat model of MI, induced by permanent ligation of the left anterior descending (LAD) coronary artery, was used for in vivo studies. All the procedures were performed with the approval of the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the *Guide for Care and Use of Laboratory Animals* (NIH publication no. 86-23). Fisher-344 rats (body weight, 250–300 g) were intubated orally, placed on a volume-cycled ventilator (Rodent Ventilator, model 683; Harvard Apparatus Inc., Holliston, MA), and maintained under general anesthesia with 1 to 2% isoflurane in air. An oblique 12-mm incision was made 8 mm away from the left sternal border toward the left armpit. The chest cavity was opened with scissors by a small incision (10 mm long) at the level of the third or fourth intercostal space, 2 to 3 mm from the left sternal border. The LAD was visualized as a pulsating bright-red spike running through the midst of the heart wall from underneath the left atrium toward apex. The LAD was ligated 1 to 2 mm below the tip of the left atrium using a tapered needle and a 6-0 polypropylene ligature passed underneath the LAD, and a double knot was made to occlude the LAD. Occlusion was confirmed by a sudden change in color (pale) of the anterior wall of the left ventricle (LV). ECG changes were recorded, and ST (ventricular depolarization) elevation was observed after LAD ligation. Multiple injections of MSCs (a total of 5×10^5 cells in 100 μl) were given in the infarct regions of the hearts 30 min after LAD ligation. The chest cavity was closed by bringing together the third and fourth ribs with one 4-0 polypropylene silk suture. The layers of muscle and skin were closed with a 4-0 polypropylene suture, and the rats were allowed to recover under a warm light.

The animals were divided into three groups, each consisting of six animals. The MI group received a sham treatment and serum-free growth medium without MSCs; the MSC group received transplanted MSCs alone, cultured under hypoxic conditions; and the MSC + TMZ group received transplanted MSCs preconditioned with TMZ under hypoxic conditions. The animals were sacrificed 4 weeks after cell transplantation. The hearts were explanted and immediately snap-frozen for Western blot studies. Hearts were also fixed in formalin for histological studies for evaluation of tissue fibrosis and key biochemical markers.

Echocardiography. Transthoracic M-mode echocardiography measurements were conducted at baseline and at 4 weeks after MSC transplantation using a GE Vivid 7 ultrasound imaging system equipped with a 13-MHz linear array transducer. Rats were anesthetized with 2% isoflurane in air for the duration of the procedure. Heart size and shape were calculated using the M-mode and two-dimensional short-axis image plane of the LV. Measurements were averaged from three cardiac cycles. The data were used to estimate percentage LV ejection fraction (EF) and fractional shortening (FS).

Measurement of Fibrosis. After measurement of hemodynamic function, the animals were euthanized, and the hearts immediately recovered and washed two to three times in cold PBS. The excised hearts were then cut into three transverse slices. Each slice was fixed in 4% paraformaldehyde and embedded in paraffin. The middle transverse section was stained with Masson-Trichrome for infarct size (fibrosis) determination. Fibrosis was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total LV epicardial and endocardial circumferences using computer-based planimetry. Quantitative assessment of each parameter was performed using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Data Analysis. The statistical significance of the results was evaluated using analysis of variance and a Student's *t* test. Values were expressed as mean ± S.D. A *p* value of <0.05 was considered significant.

Results

Effect of Hypoxia and H_2O_2 on the Viability of MSCs. The effect of hypoxic culture on the nuclear and mitochondrial viability of MSCs was studied. Cells cultured using 2% O_2 for 24 h did not show any significant change in their viability compared with normoxic (20% O_2) culture (Fig. 1, A and B). MSCs treated with H_2O_2 (100 μM) for 1 h under hypoxic conditions caused significant reductions in the nuclear (40%) and mitochondrial (44%) viabilities compared with hypoxic culture without H_2O_2 treatment (Fig. 1, C and D). The results established that culture of MSCs under hypoxic conditions had no effect up on cellular viability, but subsequent exposure to H_2O_2 was able to significantly reduce cellular viability.

Preconditioning of MSCs with TMZ Protects Cells against H_2O_2-Induced Toxicity To study the effect of TMZ pretreatment on H_2O_2-induced cytotoxicity, MSCs grown under hypoxic conditions were treated with 10 μM TMZ in culture for 6 h, followed by exposure to H_2O_2 for 1 h. At the end of treatment period, the cells were analyzed for nuclear and mitochondrial viability. As anticipated, H_2O_2 exposure resulted in a significant reduction in cell viability compared with untreated control (Fig. 2, A and B). TMZ alone had no effect on cellular viability, and cells pretreated with TMZ
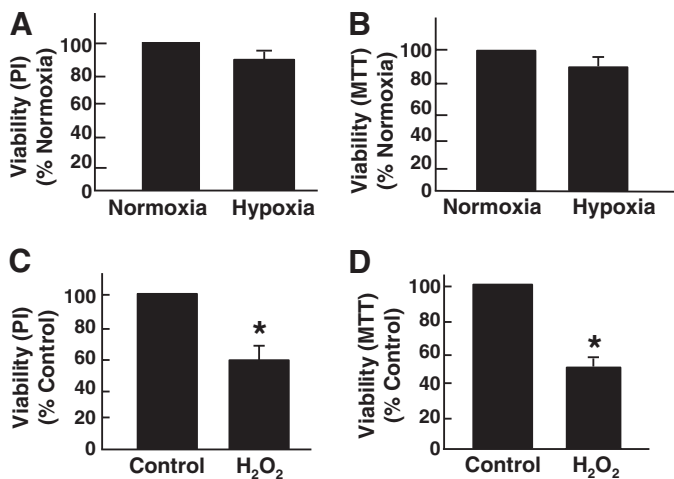
Fig. 1. Effect of hypoxia and hydrogen peroxide on the nuclear and mitochondrial viability of MSCs. Cells were cultured under normoxic (20% O_2) or hypoxic (2% O_2) conditions for 24 h. After 24 h in hypoxic culture and when the cells reached 80% confluence, H_2O_2 (100 μM) was included in the culture medium for an additional 1 h. Cellular viability was determined by MTT and PI-binding assays. The data were expressed as mean $\pm$ S.D. from three independent experiments. A, viability of MSCs determined by PI staining. There was no significant change in the viability of MSCs cultured under hypoxic conditions compared with normoxic controls. B, mitochondrial viability as determined by MTT assay. Hypoxia (2% O_2) had no significant effect on the mitochondrial viability of MSCs. C, effect of H_2O_2 on the nuclear viability of MSCs cultured under hypoxic conditions. D, effect of H_2O_2 on the mitochondrial viability of MSCs cultured under hypoxic conditions. *, $p < 0.05$ versus untreated control. Both PI and MTT assays demonstrated that 100 μM H_2O_2 was cytotoxic to MSCs cultured under hypoxic conditions.

showed a significant degree of protection against H_2O_2-induced toxicity compared with the non-TMZ control. A significantly higher level of LDH, a marker of membrane damage, was observed in the supernatant after H_2O_2 treatment, whereas this damage was significantly attenuated in cells pretreated with TMZ (Fig. 2C).

Under ischemic conditions, TMZ is known to improve mitochondrial metabolism by decreasing oxygen consumption (Monteiro et al., 2004). To check whether TMZ treatment affected oxygen consumption by MSCs under the given experimental conditions, we determined the OCR of the cells using EPR oximetry. Figure 2D shows that pretreatment with TMZ significantly reduced the OCR of MSCs. It should be noted that a similar reduction in OCR was observed in cells treated with H_2O_2, possibly because of impairment of mitochondrial function.

TMZ Preconditioning up-Regulates the Expression of Survival Proteins in MSCs. To understand the molecular mechanisms and biochemical pathways that lead to the inhibition of H_2O_2-induced damage in MSCs by TMZ exposure, we investigated the expression levels of some key hypoxic and survival marker proteins, including HIF-1α, survivin, pAkt, Akt, and Bcl-2, by Western blotting. MSCs, cultured under hypoxic conditions, were treated with TMZ for 6 h, followed by incubation with 100 μM H_2O_2 for 2 h. The expression of HIF-1α increased significantly in cells cultured under hypoxic conditions compared with the cells cultured under normoxic conditions (Fig. 3). HIF-1α expression was markedly reduced in cells not exposed to TMZ but exposed to H_2O_2. Pretreatment with TMZ resulted in a significant increase in HIF-1α expression compared with H_2O_2-treated hypoxic cells. Likewise, in cells exposed to TMZ before H_2O_2

challenge, there was an enhancement of survivin and Bcl-2 expression and increased phosphorylation (activation) of Akt. Overall, the Western blot studies indicated a marked increase in the expression of HIF-1α, survivin, Bcl-2, and pAkt in MSCs pretreated with TMZ. To further confirm whether the increased level of Bcl-2 was due to overexpression of Bcl-2, we performed RT-PCR analysis. The data (Fig. 3C) showed a substantial overexpression of Bcl-2 in preconditioned MSCs, suggesting that TMZ increased the Bcl-2 protein at the expression level.

Functional Improvement in the Infarct Hearts Transplanted with Preconditioned MSCs. Four weeks after stem cell transplantation in MI heart, cardiac functions were evaluated by M-mode echocardiography (Fig. 4). LVEF and left-ventricular FS were significantly decreased in the MI group compared with the noninfarcted control (baseline). LVEF and FS were significantly improved in the MI group treated with MSCs cultured under hypoxic conditions (MSC). An even greater recovery of cardiac function was observed in the group treated with MSCs grown under hypoxic conditions and pretreated with TMZ (MSC + TMZ).

Reduction of Fibrosis in the Infarct Hearts Transplanted with Preconditioned MSCs. Recovered heart sections stained with Masson-Trichrome showed extensive fibrosis in the MI heart (Fig. 5). The extent of fibrotic tissue was significantly reduced in the MSC group compared with the MI group. The hearts that received MSCs pretreated with TMZ showed significantly further reduction in fibrosis compared with the untreated MSC group.

Overexpression of Bcl-2 Protein in the Infarct Hearts Transplanted with Preconditioned MSCs. Western blot analysis of the explanted heart tissue showed significant increases in pAkt and Bcl-2 expression in the hearts treated with preconditioned MSCs compared with non-preconditioned MSC or MI alone hearts (Fig. 6).

Discussion

Cell survival is crucial for the success of transplantation therapy (Haider and Ashraf, 2008). It requires the adaptation of the transplanted cells to endure the hostile environment of the ischemic myocardium. We evaluated a pharmacological strategy that included preconditioning of MSCs with TMZ to make them resistant to subsequent exposure to lethal conditions upon transplantation. The major findings of our study are: 1) TMZ preconditioning significantly attenuated the H_2O_2-induced impairment of cellular viability and membrane damage in MSCs under hypoxic conditions; 2) TMZ preconditioning markedly increased the levels of cell survival proteins survivin, pAkt, and Bcl-2 in MSCs; 3) transplantation of MSCs preconditioned with TMZ significantly augmented the functional improvement and fibrosis reduction in the infarcted hearts; and 4) the infarcted hearts treated with TMZ-preconditioned MSCs showed a substantial increase in the phospho-Akt and Bcl-2 levels. The present study clearly demonstrated the potential benefits of pharmacological preconditioning of MSCs with TMZ for stem cell therapy for repairing ischemic damage in the heart.

TMZ is an antianginal drug that protects ischemic cells by restoring their ability to produce energy. In the heart, TMZ induces metabolic changes without altering the hemodynamic function (Lopaschuk et al., 2003). It optimizes cardiac
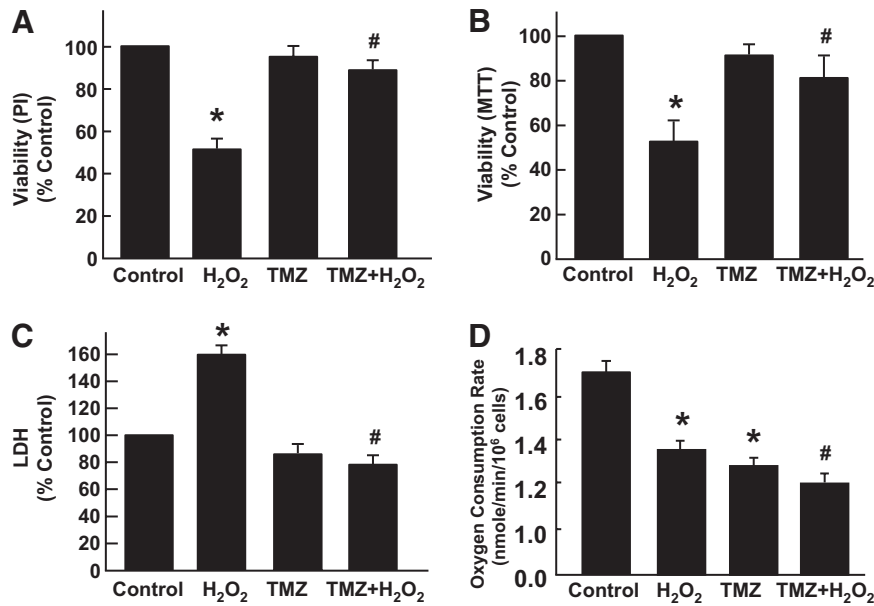
Fig. 2. Effect of TMZ on H_2O_2-induced cytotoxicity in MSCs cultured under hypoxic conditions. MSCs, kept in hypoxic culture for 24 h, were treated with 10 μM TMZ for 6 h, followed by 100 μM H_2O_2 for 2 h. The data were expressed as mean ± S.D. from three independent experiments. A, cell viability assay as determined by PI staining. H_2O_2 induced a significant reduction in the cell viability (∗, $p < 0.05$ versus control). Preconditioning of cells with TMZ significantly reversed the H_2O_2-induced cytotoxicity (#, $p < 0.05$ versus H_2O_2). B, cell viability as determined by MTT assay. H_2O_2 exposure caused a significant reduction in cell viability (∗, $p < 0.05$ versus control). Preconditioning of cells with TMZ significantly reversed the H_2O_2-induced cytotoxicity (#, $p < 0.05$ versus H_2O_2). C, membrane damage as determined by LDH assay. H_2O_2 exposure induced a significant increase in LDH level (∗, $p < 0.05$ versus control), which was attenuated by preconditioning of cells with TMZ (#, $p < 0.05$ versus H_2O_2). D, oxygen consumption in MSCs pretreated with TMZ for 6 h followed by treatment with H_2O_2 for 2 h under hypoxic conditions. Data are expressed as mean ± S.D. of OCR using five independent experiments. ∗, $p < 0.01$ versus control. #, $p < 0.05$ versus H_2O_2. Preconditioning of cells with TMZ shows a significant decrease in OCR. A significant decrease in OCR is also observed in cells treated with H_2O_2, which may be due to H_2O_2-induced impairment of mitochondrial respiration.
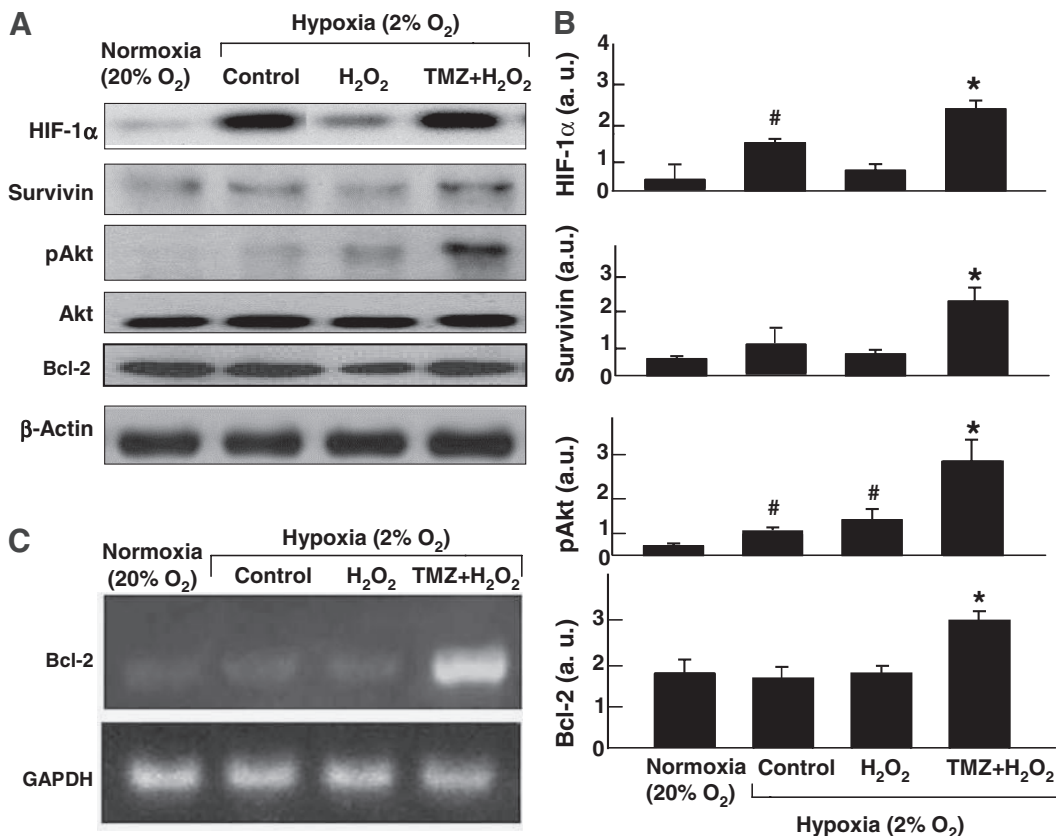

Fig. 3. Analysis of hypoxia and survival proteins expressed by the MSCs treated with TMZ and H_2O_2. Western blots were obtained from MSCs cultured under normoxia (20% O_2), hypoxia (2% O_2, control), and treatment with H_2O_2 (2 h) without or with TMZ preconditioning (6 h). A, representative blots of HIF-1α, survivin, pAkt, Akt, and Bcl-2 are shown. B, quantitative analysis of HIF-1α, survivin, pAkt, and Bcl-2 using data obtained from three different blots and expressed as mean ± S.D. #, $p < 0.05$ versus normoxia; ∗, $p < 0.05$ versus H_2O_2. TMZ preconditioning significantly increased the levels of HIF-1α, survivin, pAkt, and Bcl-2 proteins. C, RT-PCR analysis of Bcl-2 gene expression in MSCs preconditioned with TMZ followed by treatment with H_2O_2. Bcl-2 gene expression was substantially enhanced by TMZ preconditioning.
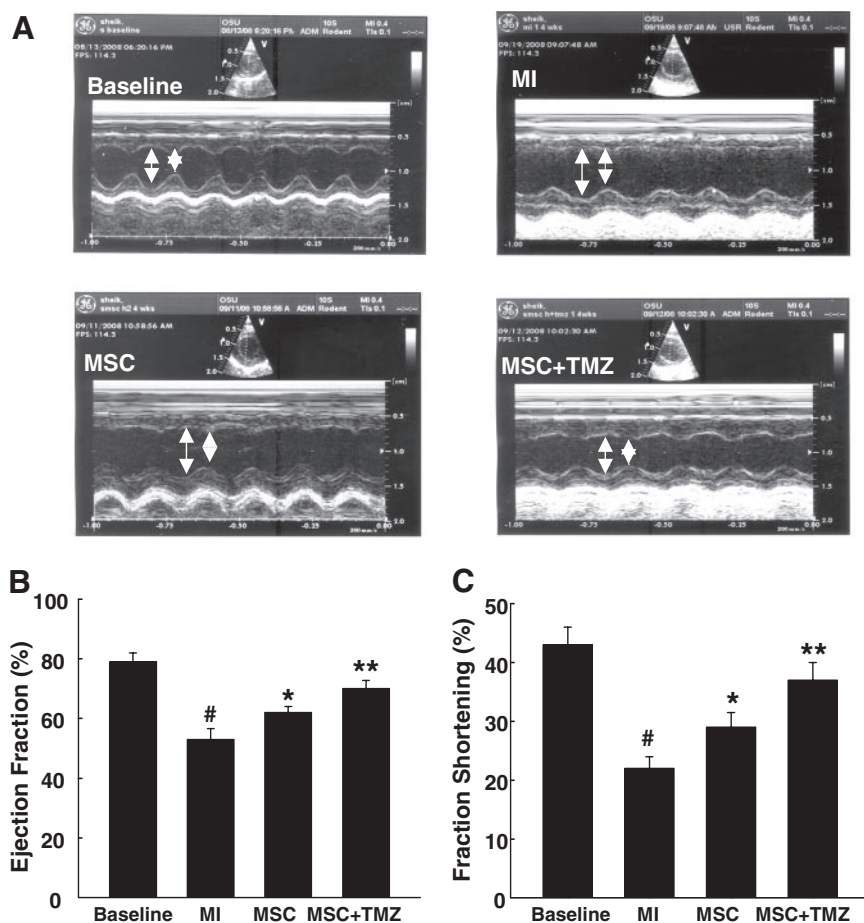
A

Fig. 4. Recovery of cardiac function at 4 weeks after MSC transplantation in the infarct heart. Transthoracic echocardiography was performed in noninfarct (baseline), infarct (MI), infarct hearts treated with MSCs (MSC), and infarct hearts treated with MSCs preconditioned with TMZ (MSC + TMZ). Representative recordings of M-mode echocardiogram (A), LV ejection fraction (B), and fraction shortening (C) are shown. Results are expressed as mean ± S.D. using six animals/group. #, $p < 0.05$ versus baseline; ∗, $p < 0.05$ versus MI; ∗∗, $p < 0.05$ versus MSC. The MI hearts treated with TMZ-preconditioned cells had greater functional recovery than those with non-preconditioned cells.

B

C

metabolism by reducing fatty acid oxidation through selective inhibition of 3-ketoacyl CoA thiolase enzyme in the mitochondria of cardiomyocyte. As a result, TMZ attenuates the adverse effects of free fatty acid-associated oxidative stress (Gambert et al., 2006), lessens oxygen demand by decreasing oxygen consumption (Monteiro et al., 2004), and improves
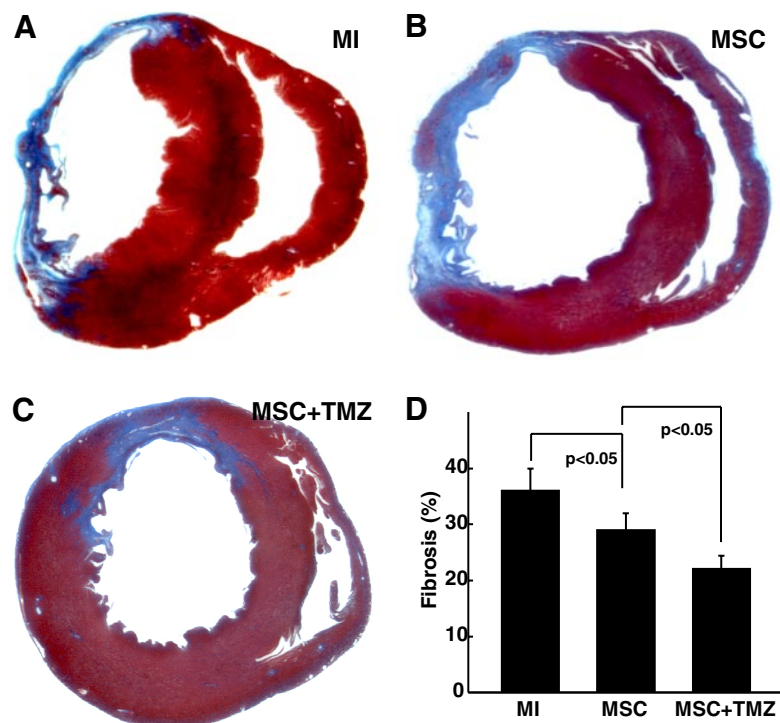
A MI

B MSC

C MSC+TMZ

D

Fig. 5. Effects of preconditioning on tissue fibrosis 4 weeks after MSC transplantation. Masson-Trichrome staining of heart sections was performed in infarcted hearts (MI) and infarcted hearts treated with non-preconditioned MSCs (MSC) and MSCs preconditioned with TMZ (MSC + TMZ) before transplantation. A to C, representative images of heart sections stained with Masson-Trichrome. D, percentage of fibrosis in hearts 4 weeks after transplantation, as determined by computer planimetry. Data are expressed as mean ± S.D. using six hearts per group. Hearts treated with MSCs preconditioned with TMZ exhibit a significant reduction in fibrosis compared with the MSC group.
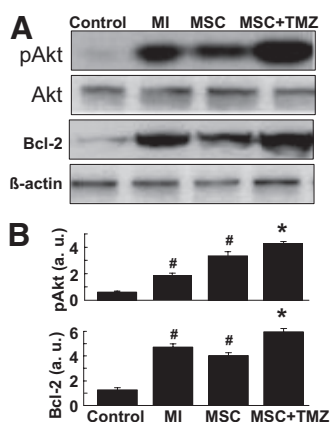
Fig. 6. Protein expression in the explanted tissue of hearts treated with MSCs preconditioned with TMZ under hypoxic conditions. A, representative Western blot images showing pAkt, Akt, and Bcl-2 levels. B, quantitative analysis of pAkt and Bcl-2 proteins in the explanted tissue. Values are expressed as mean ± S.D. obtained from six blots. ∗, $p < 0.05$ versus MI or MSC groups. Hearts treated with TMZ-preconditioned MSCs show a significantly enhanced Akt and Bcl-2 expressions.

mitochondrial metabolism and cardiac performance during ischemia (Kantor et al., 2000). At the cellular level, TMZ preserves ATP production, reduces the generation of oxygen free radicals (Maupoil et al., 1990; Gambert et al., 2006; Kutala et al., 2006), and reduces intracellular acidosis and calcium overload (Kantor et al., 2000). TMZ has been shown to protect hearts from ischemia-induced electrical dysfunction leading to ventricular fibrillation (Vaillant et al., 2008), ischemia-reperfusion-induced damage to mitochondrial respiration (Guarnieri and Muscari, 1993), and ischemia-reperfusion injury by decreasing myocardial lactate content early at reperfusion (Pantos et al., 2005). Tritto et al. (2005) demonstrated that TMZ attenuated tissue injury in postischemic hearts by inhibiting the activation of neutrophils. We reported recently that pretreatment of hearts with TMZ significantly enhanced the functional recovery by combined effects of antioxidant and anti-ischemic activities and enhanced prosurvival Akt activity (Kutala et al., 2006).

MSCs, such as used in this study, are derived from hypoxic niches in bone marrow, where the pO_2 is quiet low. Usually, in vitro expansion and incubation of MSCs are carried out under normoxic (20% O_2) conditions to achieve high cellular vitality and proliferation rates. However, when these cells are transplanted in the infarct tissue, they encounter severe hypoxic conditions, typically less than 0.5% O_2 (Khan et al., 2007, 2008; Mohan et al., 2009), which can induce apoptosis and cell death. This hypoxia-induced apoptosis and cell loss can be prevented by hypoxic preconditioning, that is, by exposure of MSCs to less severe hypoxic conditions (1–3% O_2) for a period of time before transplantation into the ischemic heart (Hu et al., 2008; Rosová et al., 2008; Wang et al., 2008). Since the focus of this work was to study the effect of TMZ on the survival of transplanted MSCs under the hypoxic and oxidative environments in the ischemic heart, we used cells that were cultured and treated under hypoxic (2% O_2) conditions. We did not find significant change in the viability of MSCs cultured under hypoxic conditions, compared with normoxic culture. However, hypoxic exposure significantly increased the HIF-1α level and induced Akt activation, which is known to promote cell survival by inhibiting apoptosis. Rosová et al. (2008) have demonstrated that human bone

marrow-derived MSCs cultured under hypoxia (2% O_2 for 16 h) maintained their viability and cell cycle rates by activating the Akt signaling pathway. Hypoxic preconditioning of rat MSCs (0.5% O_2 for 6 h) has been shown to attenuate hypoxia reoxygenation-induced apoptosis by stabilizing mitochondrial membrane potential, up-regulating Bcl-2, vascular endothelial growth factor, and promoting extracellular signal-regulated kinase and Akt phosphorylation (Wang et al., 2008).

Bcl-2 is an antiapoptotic protein originally found to be overexpressed in B-cell lymphoma. It is a critical inhibitor of apoptotic cell death in ventricular myocytes. In the ischemic heart, Bcl-2 contributes to cardiac protection by regulating the metabolic functions of mitochondria (Kirshenbaum and de Moissac, 1997). The role of Bcl-2 protein in myocardial stem cell therapy has been reported. Li et al. (2007) genetically modified adult rat bone marrow-derived MSCs to overexpress Bcl-2 and demonstrated substantial resistance of the transplanted cells to apoptosis and remarkable functional recovery in an acute model of myocardial infarction in rats. A recent report by Hu et al. (2008) demonstrated increased expression of prosurvival and proangiogenic factors, including HIF-1α and Bcl-2 by hypoxic preconditioning of mouse MSCs. They further showed that transplantation of the hypoxic-preconditioned MSCs in the infarcted mouse hearts resulted in increased angiogenesis and enhanced morphologic and functional benefits of stem cell therapy. Our in vitro results showed a significantly increased expression of Bcl-2 in TMZ-preconditioned MSCs subjected to H_2O_2-induced oxidant stress, suggesting that TMZ increased the Bcl-2 protein at the expression level. A significantly higher level of Bcl-2 was also observed in the hearts treated with the TMZ-preconditioned MSCs. However, the precise role and mechanism by which TMZ regulates Bcl-2 expression is yet to be understood.

This is the first report on the use of TMZ for preconditioning of cells. However, there is abundant literature on the use of TMZ for myocardial preconditioning (Opie, 2003; Argaud et al., 2005; Kara et al., 2006). TMZ is reported to inhibit mitochondrial permeability transition pore opening and protect the rabbit heart from prolonged ischemia-reperfusion injury (Argaud et al., 2005). Preconditioning of myocardium with TMZ has been shown to protect the heart against ischemia-induced arrhythmias, reduce myocardial infarct size, preserve the effects of ischemic preconditioning and pharmacological preconditioning, and mimic ischemic preconditioning in anesthetized rats (Kara et al., 2004). In the present study, we transplanted MSCs preconditioned with TMZ, but we did not treat the animals with TMZ, either pre- or posttransplantation. Thus, the present study does not establish whether the beneficial effects of transplantation of TMZ-preconditioned MSCs is entirely due to TMZ. Further studies using treatment of animals with TMZ are required to delineate the mechanism of protection by TMZ in vivo. In addition, it remains to be established whether the preconditioning effect observed in the present study is actually due to the shift toward glucose metabolism or to other effects of TMZ. Because the MSCs are physiologically exposed to low oxygen tension in bone marrow, a prevalence of glycolysis over mitochondrial respiration could actually be the normal condition for these cells. Hence, it is possible that TMZ is exerting its beneficial effect, not so much because it induces a new

phenotype, but rather because it may help preserving/restoring the glucose-avid phenotype congenial to these cells. Furthermore, it has long been known that glycolytically generated ATP is preferentially employed in driving a number of chemical processes occurring at membrane level (Nakamura et al., 1993; Cappelli-Bigazzi et al., 1997). Thus, favoring glycolysis may have a favorable impact on a variety of functions of stem cells.

In summary, MSCs preconditioned with TMZ showed a significant protection against H_2O_2-induced loss of cellular viability, membrane damage, and oxygen metabolism accompanied by a significant increase in HIF-1α, survivin, pAkt, and Bcl-2 protein levels and gene expression. A significant improvement in the recovery of myocardial function, decrease of tissue fibrosis, and up-regulation of pAkt and Bcl-2 levels were observed in infarcted heart tissues treated with TMZ-preconditioned cells. This study clearly demonstrated the potential benefits of pharmacological preconditioning of MSCs with TMZ for stem cell therapy for repairing myocardial ischemic damage.

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